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(57) Abstract

A method and reagent system are disclosed for the isolation, identification and/or analysis of selected populations in heterogeneous samples. The method and reagent system of this invention has application to any environment in which the accurate study and/or analysis of cells requires their isolation or enrichment. One of the environments in which this invention can be used to advantage is in the analysis of tumor cells by a fluorescence measuring instrument such as a flow cytometer.

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SOLID TUMOR ANALYSIS BY MULTIPARAMETRIC FLOW CYTOMETRY

This application relates to application docket number 139,647, entitled Viability Probes for Isolation, Identification and/or Analysis of Cells, filed concurrently herewith. Said application is herewith incorporated by reference.

Technical Field

The present invention relates to biological testing and in particular to methods for identifying and analyzing biological parameters of selected subpopulation of cells in a heterogeneous sample. The methods of the invention facilitate analysis of complex biological samples, such as solid tumors, by means of staining the cells with a cocktail of biological probes informative for cellular parameters, such as viability, tissue type, tumor-associated antigen expression, proliferation, and deoxyribonucleic acid (DNA) content.

Background Art

Since its relatively recent introduction, flow cytometry has become an invaluable tool in the study of cells. Presently known and available flow cytometers useful for detecting particles, cells and the like, commonly include one or more light sources (usually lasers) and photoelectric detectors for the measurement of fluorescence and light scatter. Light scatter patterns and fluorescence emission of fluorescent labels, generated by the incidence of light upon cells in a continuously flowing stream, can be used to distinguish subpopulations of cells in a cell mixture.

One of the earliest applications of flow cytometry was the analysis of cellular DNA content and proliferative capacity. In the field of oncology, much effort has been devoted to the correlation of these parameters with

disease prognosis and treatment, e.g., Barranco, et al., Cancer Research, Vol. 42, pgs.1 2894-2898 (1982).

Another early application of flow cytometry was immunophenotyping, the characterization of cells by staining with fluorochrome-conjugated antibodies directed against specific antigenic determinants. Initially immunophenotyping was limited by instrumentation, fluorophore availability, and antibody production technology to single markers.

While both DNA content and the expression of a single marker are important pieces of information, they are of limited utility in the analysis of complex biological samples such as solid tumors. First because cell function is a highly interactive process, an understanding thereof is proportional to the number of markers analyzed and correlated. Second, solid tumors may contain dozens of normal cell types in addition to the actual tumor cells, as well as large numbers of dead cells. In sufficient numbers, these "contaminating" cells can completely obscure the tumor cells. Thus, accurate analysis of target cells depends on isolation, or enrichment, of the target population, i.e. live tumor cells, from other cell types. Electronic gating on the basis of light scatter is the most common technique for isolating target cell populations. Different types of cells will scatter incident light differently when they flow through the laser beam in the flow cytometer, and in samples with few cell types, light scatter differences can often be used to distinguish one cell type from another. The target cell population can then be isolated for analysis by electronic gating, i.e., bitmapping the relevant population(s) in a light scatter histogram. For a summary on gating see Bauer et al., Clinical Flow Cytometry, Principles and Applications, Chapter 5, pgs. 87-88, (William and Wilkins, 1993). However, attempts to separate cell populations by electronic gating on the basis of light scatter are only marginally successful in solid tumors because of marked

pleomorphism. These technical difficulties are more characteristic of solid tumor than hematopoietic tumor analysis because of the generally greater heterogeneity of solid tumors and the cellular damage caused by mechanical and chemical dissociation of solid tumors into the single cell suspensions required for flow cytometry.

With the recent proliferation of monoclonal antibodies and fluorescent dyes, and advances in instrumentation, multiparametric flow cytometry came into being. The simultaneous analysis and correlation of multiple markers provides an alternative method of target cell enrichment. The correlation of multiple parameters also enhances our understanding of cell biology, and has far-reaching diagnostic and prognostic implications. Where once analysis was limited to the expression of a single marker or measurement of DNA content, investigators can now correlate antigen expression with cell type, proliferation, ploidy, viability, etc. The analytical potential of this technique is enormous, due to the virtually countless combinations of available markers.

Zarbo et al., Analytical and Quantitative Cytology and Histology, 11:391-402 (1989), disclose the use of a tissue-specific marker to isolate solid tumor cells for DNA analysis. However, present technology has been generally restricted to the simultaneous analysis of relatively few markers, due to instrument limitations, fluorophore availability, and technical difficulties, such as those associated with low viability and combined surface and intracellular staining.

A need exists, therefore, for improved methods to determine the presence and biological characteristics of selected subpopulations of cells in heterogeneous samples, particularly those, such as solid tumors, containing large numbers of dead cells, which can significantly distort results by nonspecifically binding biological probes.

The present invention overcomes the above problems by (1) significant improvement in accuracy of analysis due to

the elimination of non-target and dead cells, which can significantly interfere with analysis, and (2) enhancement of the analytical power of flow cytometry by the correlated analysis of five markers.

Disclosure of the Invention

Broadly, the invention is a method for isolating target cell populations, i.e., live tumor cells, by electronically gating out, or excluding, dead and non-target cell populations on the basis of differential staining by the various markers. The live tumor cell population can then be analyzed for proliferative status, DNA content, and tumor associated antigen expression.

Accordingly, it is an object of the present invention to provide a method for isolation or enrichment of cells for analysis of complex biological tissues.

It is another object of the present invention to provide a method for isolation or enrichment of cells for analysis of complex biological tissues, wherein the cells are viable tumors cells.

It is an additional object of the present invention to provide a novel method of eliminating non-tumor and dead cells from a biological sample.

It is another object of the present invention to measure the DNA content and proliferation in cells of a heterogenous sample.

It is another object of the present invention to provide a panel of markers which are useful in staining cells of a biological sample.

Yet another object of the present invention is to provide a method for amplifying flow cytometric analytical capabilities by the correlation of five markers.

It is another object of the present invention to provide a flow cytometric technique for simultaneous measurement of five fluorophores.

Yet another object of the present invention is to provide a method for electronically gating out dead cells

and non-target cells populations in heterogeneous samples, by differential staining and "progressive gating" with the markers of the present invention and/or by differential light scatter, using a series of progressive gates.

It is another object of the present invention to significantly improve the accuracy of analysis by the isolation and enrichment of target cells in a heterogeneous sample.

It is another object of the present invention to correlate the parameters of the present invention with treatment and/or response to drug therapy.

It is another object of the present invention to correlate the parameters of the present invention with treatment and/or response to cancer therapy.

It is another object of the present invention to provide a kit for the isolation or enrichment of cells for analysis of complex biological tissues, the kit including containers of each marker comprising (1) a viability marker, (2) a tumor-associated antigen, (3) a tissue-specific antigen, (4) a proliferation-associated antigen, and (5) a DNA-content dye.

Additional objects, advantages and novel features of the invention will be set forth in part in the description which follows and in part will become apparent to those skilled in the art upon examination of the following or may be learned by practice of the invention. The objects and advantages of the invention may be realized and attained by means of the instrumentalities and combinations particularly pointed out in the appended claims.

Brief Description of Drawings

The preferred embodiments of this invention will now be described by way of example, with reference to the drawings accompanying this specification in which:

Figure 1 diagrams the concept of "progressive gating," demonstrating the sequential application of multiple gates.

Figures 2 A-H comprise an ungated histogram display of all parameters acquired from a mixture of cells (25% live MDAs, 25% dead MDAs, 25% CEMs, and 25% lymphocytes) and stained with a cocktail of MC5-RD1, CK-ECD, PCNA-APC, EMA (viability probe), and DAPI.

Figures 3 A-H comprise a viability EMA-gated display of the cell mixture defined in Figs. 2 A-H and stained with the cocktail defined in Figs. 2 A-H.

Figures 4 A-H comprise a viability- and cytokeratin-gated display of the cell mixture defined in Figs. 2 A-H and stained with the cocktail defined in Figs. 2 A-H.

Figures 5 A-H comprise a viability-, cytokeratin-, and tumor-associated antigen (MC5)-gated display of the cell mixture defined in Figs. 2 A-H and stained with the cocktail defined in Figs. 2 A-H.

Figures 6 A-H comprise a viability-, cytokeratin-, tumor-associated antigen- (MC5)-gated, and aggregate display of the cell mixture defined in Figs. 2 A-H and stained with the cocktail defined in Figs. 2 A-H.

Figures 7 A-H comprise an ungated histogram display of all parameters acquired from a breast tumor stained with a cocktail of MC5-RD1, CK-ECD, PCNA-FITC, 7-AAD (viability probe), and DAPI.

Figures 8 A-H comprise a viability (7-AAD)-gated display of the tumor defined in Figs. 7 A-H and stained with the cocktail defined in Figs. 7 A-H.

Figures 9 A-H comprise a viability- and cytokeratin-gated display of the tumor defined in Figs. 7 A-H and stained with the cocktail defined in Figs. 7 A-H.

Figures 10 A-H comprise a viability-, cytokeratin-, and tumor-associated antigen (MC5)-gated display of the

tumor defined in Figs. 7 A-H and stained with the cocktail defined in Figs. 7 A-H.

Figures 11 A-H comprise a viability-, cytokeratin-, and tumor-associated antigen (MC5)-gated and aggregate display of the tumor defined in Figs. 7 A-H and stained with the cocktail defined in Figs. 7 A-H.

Figures 12 A-D represent the laser and optical filter configurations for Panels I-IV (infra).

Modes for Carrying Out the Invention

While the present invention is satisfied by embodiments in many different forms, herein will be described in detail a particular embodiment of the invention, with the understanding that the following description is to be considered as exemplary of the principles of the invention and is not intended to limit the scope of the invention as determined by the appended claims and their equivalent.

The present invention provides for a method using multiparametric analysis of biological samples using a panel which includes various markers. The use of "progressive" electronic gating results in limitation of analysis of DNA and proliferative status to live tumor cells by generation of histograms gated on viability, tissue-specific, and disease-associated markers.

The panel of the present invention comprises (1) a viability marker, (2) a tumor-associated antigen (e.g., epithelial mucin antigen [MC5]), (3) a tissue-specific antigen (e.g., cytokeratin [CK]), (4) a proliferation-associated antigen (e.g., proliferating cell nuclear antigen [PCNA]), and (5) a DNA-content dye (e.g., DAPI).

The method of the invention includes a step of preparing the cells for measurement on a fluorescence measuring instrument, preferably a flow cytometer. Basically, such steps are as follows:

(a) aliquot cells, centrifuge and decant;

(b) stain cells with viability probe, then wash;

(c) if viability dye requires indirect label, such as in Panels II and IV, stain with secondary antibody, wash, then stain cells with unconjugated mouse antibody and wash;

(d) stain cells with tumor associated antigen and wash twice;

(e) incubate in a permeabilization/fixation agent, preferably lysolecithin/1% paraformaldehyde, for 2 minutes, centrifuge and decant;

(f) incubate in a dehydration agent, preferably methanol, for 10 minutes, centrifuge and decant;

(g) incubate in a detergent, preferably NP40, for 5 minutes;

(h) centrifuge and decant;

(i) stain cells with proliferation associated antigen marker and wash;

(j) resuspend sample in DNA dye and incubate about 20 minutes; and

(k) analyze using a flow cytometer.

The principles and operation of flow cytometers are well-established, and several flow cytometers suitable for use with the invention are available commercially. See Flow Cytometry and Sorting, Melamed et al., Second Edition, (John Wiley & Sons, New York, 1990).

The techniques of the present invention were utilized in flow cytometry instruments such as the EPICS® Elite flow cytometer available from Coulter Corporation. Such instruments employed the principle of cells moving in a columnar stream bounded by a stream of sheath fluid, such that cells flowed in single file through a laser beam. Light scatter and/or fluorescence signals from the cells were then utilized in classifying cell populations. The development of instrumentation and fluorochromes for multiparameter analysis is further described by Melamed et al., supra, Chapters 1, 2 and 12.

The techniques of gating can be used to analyze selected cell populations. In the present invention, a preferred method of gating is as follows: Live cells are gated first on the basis of the viability dye. Live cells that express the antigen specific to the tissue type that gave rise to the tumor are then gated. Live cells of that tissue type that express the tumor-associated antigen are then gated. Finally, one can analyze the DNA content and proliferative status of only these live tumor cells, thus greatly enhancing the accuracy of analysis.

Materials

Phosphate-buffered saline, lysophosphatidyl choline, Nonidet P-40 (NP40), 7-AAD, and DAPI were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum and absolute methanol were purchased from HyClone (Logan, UT) and JT Baker, Inc. (Phillipsburg, NJ), respectively. Ficoll-hypaque was purchased from Pharmacia LKB Biotechnology (Piscataway, NJ). Paraformaldehyde was obtained from Fluka Chemie A.G. (Buchs, Switzerland). Anti-cytokeratin monoclonal antibody (conjugated to either RD1 or ECD), anti-MC5 antibody (conjugated to either RD1 or APC), anti-PCNA antibody (conjugated to either FITC, APC or CY5), and matched isotype controls were provided by Coulter Corporation (Miami, FL). Anti-α-tubulin was purchased from Zymed Laboratories, Inc. (San Francisco, CA). FITC-conjugated sheep-anti-mouse $F(ab')_2$ fragments (SAM-FITC) were obtained from Silenus Laboratories (Victoria, Australia). Ethidium monoazide bromide (EMA) was purchased from Molecular Probes, Inc. (Eugene, OR). RPMI and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Biowhittaker (Walkersville, MD).

The abbreviations used in the present application and in the claims, unless otherwise indicated are as follows:
CK-ECK: cytokeratin-energy coupled dye;
PCNA-APC: proliferating cell nuclear antigen-allophycocyanin;

EMA: ethidium monoazide;
DAPI: 4',6-Diamidino-2-phenylindole;
CK: cytokeratin;
7-AAD: 7-aminoactinomycin D;
PBS: Phosphate-buffered saline;
FBS: Fetal bovine serum;
$F(ab')_2$: antigen-binding site fragments;
PCNA-CY5: proliferating cell nuclear antigen-cyanine 5;
APC: allophycocyanine;
RD1: red dye 1;
FITC: fluorescein isothiocyanate;
ECD: energy-coupled dye;
PI: propidium iodide;
MEM: Modified Eagle Medium;
MDA: M.D. Anderson, the cancer center from where the cell line originated.

Methods

Cell Preparation

Three types of samples were utilized in the optimization and application of this invention: (1) homogeneous samples of cultured cells of the MDA-MB-175-VII breast cancer cell line; (2) a cell mixture designed to simulate a breast tumor, including equal numbers of live and dead cells of the MDA-MB-175-VII breast cancer cell line, live cells of the CEM T-cell leukemic cell line, and ficoll-separated lymphocytes from normal whole blood; and (3) solid breast tumors, mechanically dissociated into single cell suspension.

Monolayer cultures of the MDA-MB-175-VII breast cancer cell line (American Type Culture Collection, Rockville, MD) were grown in high glucose DMEM supplemented with 10% fetal bovine serum at 37° C and 5% carbon dioxide (CO_2) in a humidified atmosphere. The cultures were maintained in log phase growth. Suspension cultures of CEMs (American Type Culture Collection, Rockville, MD) were grown in RPMI supplemented with 10%

fetal bovine serum at 37°C and 5% CO_2 in a humidified atmosphere. The cultures were maintained in log phase growth. For cell mixtures containing dead cells, starvation was induced by maintaining harvested cells, tightly capped, at 37° C and 5% CO_2 in a humidified atmosphere, to simulate the internally hypoxic environment of a solid tumor. Lymphocytes were separated from whole blood by the following procedure. Whole blood was diluted with an equal volume of PBS and mixed by pipet. Up to 8 Ml of the diluted blood was overlaid on 4 Ml of room-temperature ficoll-hypaque in 15 Ml polystyrene tubes. The gradients were centrifuged at 400 x g for about 30 minutes. The mononuclear layers were removed by pipet and placed in clean 15 Ml polystyrene tubes. The tubes were filled with PBS and 2.5% FBS (PBSF) and centrifuged at 400 x g for about 10 minutes at room temperature. The supernatant was decanted, and the tubes were filled again with PBSF and centrifuged at 300 x g for about 10 minutes, at 4°C. The last wash was repeated. The cells were resuspended in a small volume of PBS and counted. The volume was then adjusted with PBS to achieve the desired cell concentration. Solid tumors were mechanically dissociated into single cell suspensions, by standard procedure, as follows: slicing the specimen with a scalpel in a media dish containing about 10 mL isotonic media (e.g., Modified Eagle Medium (MEM)), and filtering the resultant cell suspension nylon mesh filters of successively decreasing diameters, from 210 to 44. The tube was centrifuged at 400 x g for about 10 minutes at room temperature, and the supernatant was poured off. The tube was then filled with MEM + 10% FBS and centrifuged again at 400 x g for about 10 minutes at room temperature and the supernatant was poured off. The cells were resuspended in a small volume of PBS and counted. The volume was then adjusted with PBS to achieve the desired cell concentration.

Cell Staining

Cells were aliquoted, at 1 x 10^6 cells per test, into 12 x 75 mm siliconized glass test tubes. The samples were centrifuged at 500 x g for about 5 minutes at room temperature. The supernatant was decanted and the tubes were blotted. 200 μL of working dilution of tubulin (or other viability marker; dose established by titration) in PBS + 2.5% FBS (PBSF) were added to each test sample and 200 μL of PBSF were added to controls. The samples were vortexed and incubated for about 15 minutes at room temperature and in room light, except for EMA-stained samples, which were exposed to fluorescent light, at a distance of about 20 cm, for about 15 minutes. 2mL PBS were added to each tube and the tubes were centrifuged at 500 x g for about 5 minutes at room temperature. The supernatant was decanted and the tubes were blotted. 200 μL of working dilution of SAM-FITC (or alternate secondary label if the viability marker was not directly conjugated; dose established by titration) in PBSF was added to each test sample, and 200 μL of PBSF were added to controls. The samples were vortexed, and incubated for about 15 minutes at room temperature, protected from light. 2 mL PBS were added to each tube and the samples were centrifuged at 500 x g for about 5 minutes at room temperature. The supernatant was decanted and the tubes were blotted. 200 μL of unconjugated isotype control (at manufacturer's recommended dose) were added to each tube to block any free antibody binding sites remaining on the SAM-FITC. The samples were vortexed and incubated for about 15 minutes at room temperature. 2 mL PBS was added to each tube and centrifuged at 500 x g for 5 minutes at room temperature. The supernatant was decanted and the tubes were blotted. 200 μL of working dilution of fluorophore-conjugated MC5 (or other tumor-associated antigen; dose established by titration) were added to each test sample, and 200 μL of isotype control (manufacturer's recommended dose) conjugated to the same fluorophore were

added to the controls. The samples were vortexed and incubated for about 15 minutes at room temperature. 2 mL PBS were added to each tube and the tubes were centrifuged at 500 x g for about 5 minutes at room temperature. The supernatant was decanted and the tubes were blotted. The wash was repeated. 1 mL of room-temperature 20 μg/mL lysophosphatidyl choline in 1% paraformaldehyde was added to each tube. The tubes were vortexed, incubated for about 2 minutes at room temperature, then centrifuged at 500 x g for about 5 minutes at room temperature. The supernatant was decanted and the tubes were blotted. 1 mL -20° C absolute methanol was added to each tube. The tubes were vortexed, incubated on ice for about 10 minutes, then centrifuged at 500 x g for about 5 minutes at room temperature. The supernatant was decanted and the tubes were blotted. 1 mL 4° C 0.1% NP40 was added to each tube. The tubes were vortexed, incubated on ice for about 5 minutes, then centrifuged at 500 x g for about 5 minutes at room temperature. The supernatant was decanted and the tubes were blotted. 200 μL of a 50 μg/mL working solution of fluorophore-conjugated PCNA (or other proliferation-associated antigen; dose established by titration) were added to each test sample, and 200 μL of isotype control (manufacturer's recommended dose) conjugated to the same fluorophore were added to the controls. The samples were vortexed and incubated for about 15 minutes at room temperature. 2 mL PBS were added to each tube and the tubes were centrifuged at 500 x g for about 5 minutes at room temperature. The supernatant was decanted and the tubes were blotted. 1 mL of a 1.5 μg/mL working solution of DAPI was added to each tube. The tubes were vortexed, and incubated for about 20 minutes at room temperature, protected from light. Finally, the samples were analyzed by flow cytometry.

Panels

The panel of the present invention consists of

(1) a viability marker, (2) a tumor-associated antigen, (3) a tissue-specific antigen, (4) a proliferation-associated antigen, and (5) a DNA-content dye.

Preferred panels for use in the present invention are represented by Panels I-IV below.

PANEL I

MC5-RD1 (tumor-associated antigen)
CK-ECD (tissue-specific antigen)
PCNA-APC (proliferation-associated antigen)
EMA (viability dye)
DAPI (DNA-specific dye)

PANEL II

MC5-RD1
CK-ECD
PCNA-FITC
7-AAD (viability dye)
DAPI

PANEL III

MC5-APC
CK-RD1
(PCNA-CY5)
Tubulin-SAM-FITC (viability marker)
DAPI

PANEL IV

MC5-RD1
CK-ECD
PCNA-CY5
Tubulin-SAM-FITC
DAPI

In the context of the present invention, monoclonal antibodies are used as marking agents. A tagging agent

comprises a fluorescence-emitting agent (fluorophore) which alone or in conjugation to a marking agent can label a structure(s) in or on a cell.

Fluorophores useful in the invention include, but are not limited to, nucleic acid-specific dyes, phycobiliproteins, cyanine derivatives, fluorescein derivatives, rhodamine derivatives, or any combination of the above.

Examples of marking agents are listed on Table 1 below.

TABLE 1

DYE	LASER	EXCITATION (MAX)	EMISSION (RANGE 50%)
FITC	488	495	520 (505-540)
RD1[1] (RPE)	488	565, 496	576 (565-587
ECD[2] (PE + Texas Red)	488	565, 496	613 (595-635)
PI	488	520	610 (560-680)
EMA[3]	488	510	600
Cyanine 5 (CY5)	633	651	665 (650-685)
APC	633	652	660 (648-672)
DAPI	UV	348	470 (440-520)
7-AAD	488	550	660 (620-710)

[1] Red Dye 1 is Coulter's name for R-PE which is R-phycoerythrin.
[2] ECD is a tandem conjugate of PE and Texas Red.
[3] EMA is used here as a viability dye

Flow Cytometry

Laser and Optical Filter Configurations

Samples were analyzed on an EPICS® Elite flow cytometer (Coulter Corporation, Miami, FL) configured with five photomultiplier tubes and three lasers: a water-cooled, 5 Watt (W) argon laser, an air-cooled 488 nanometer (nm) argon laser operated at 15 milliwatt (mW), and a 10 mW air-cooled 633 nm helium-neon laser. The laser and filter configurations for each of Panels I-IV given in Figures 13 A-D, respectively.

In all but one of the panels, three lasers were used for excitation; in the remaining panel, two lasers were used. The lasers were spatially separated as indicated in the preceding schematics, where two lines drawn closely together and annotated with two wavelengths joined by a "+" represent colinear lines, and the lower line(s) is (are) separated from the upper line(s) by a distance of 40 μsec. In all cases, time-gated amplification of signals was used to permit acquisition of data from two fluorophores (each fluorophore excited by one of two spatially separated lasers) on each of two of the fluorescence channels. Specifically, DAPI and FITC signals were detected, with gated amplification, by the same channel (with reflection by a 550 nm dichroic longpass filter and transmission by a 525 nm bandpass filter); APC and ethidium monoazide were detected, with gated amplification, by the same channel (with reflection by a 600 nm dichroic longpass filter and transmission by a 635 nm bandpass filter); and APC and CY5 were detected, with gated amplification, by the same channel (with reflection by a 650 nm longpass filter and transmission by a 675 nm bandpass filter). Fluorescence emission of RD1 was reflected by a 600 or 650 nm dichroic longpass filter and transmitted through a 575 nm bandpass filter. Fluorescence emission of ECD was transmitted through a 600 nm dichroic longpass filter and transmitted through a 610 nm bandpass filter. Fluorescence emission of 7-aminoactinomycin D was transmitted through 650 nm dichroic longpass and 675 nm bandpass filters. CY5, when not

combined with APC, was reflected by a 660 nm dichroic shortpass filter and transmitted through a 675 nm bandpass filter. Autofluorescence of unstained controls was measured through the same filter configurations as the stained samples with which they were matched.

Instrument Calibration

The flow cytometer was calibrated according to the method described in the instrument manual.

Data Acquisition and Display

Eight or nine parameters were acquired, depending on which cocktail was used: forward light scatter, side light scatter, five or six fluorescence signals (in some configurations, both peak and integral signals were collected for DAPI), and PRISM. At least five thousand events were collected in listmode format for each sample, with linear amplification of forward and side scatter, and four-decade logarithmic amplification of all fluorescence signals except DAPI, which was acquired as an integral, and in some configurations, also a peak signal. Acquisition and analysis were performed with the Elite software. The Elite flow cytometer permits a maximum display of eight histograms. A combination of eight one- and two-parameter histograms was displayed such that each parameter was displayed in at least one histogram.

Gating

A common feature of flow cytometers is the ability to "gate" computerized data displays and analyses. Gates (rectilinear regions or bitmaps in a 2-parameter histogram or rectilinear regions in a 1-parameter histogram, encompassing selected populations) are drawn in the histogram displays, establishing channel boundaries corresponding to upper and lower limits of signal amplitude of fluorescence and/or light scatter (and/or any other parameters used). Upon restarting data acquisition

after drawing the gates, only data within the gates (or outside the gates, depending on the nature of the gate equation) will be acquired and displayed.

In this application, we performed a technique of "progressive gating," whereby a series of gates was applied, using isotype controls (and in the case of viability probes, unstained cells) to establish background. The first gate was drawn in the viability histogram such that only viable cells were included. (Figures 3 A-H and 8 A-H). The viability gate was then applied to the remaining histogram displays, and after restarting data acquisition, a gate was drawn in the cytokeratin histogram (Figures 4 A-H and 9 A-H), such that only cytokeratin-positive cells (i.e., cells of epithelial origin, which gives rise to breast tumor cells) were included. This gate, in addition to the viability gate, was then applied to the remaining histogram displays, and after restarting data acquisition, a gate was drawn in the MC5 histogram (Figures 5 A-H and 10 A-H), such that only those cells positive for MC5 staining were included. This gate, in addition to the viability and cytokeratin gate, was then applied to the remaining histogram displays of DAPI (where applicable, both peak and integral signals) and PCNA. In those configurations in which both the peak and integral DAPI signals were collected, a fourth gate was drawn in the histogram juxtaposing these two signals to exclude events (i.e., aggregates) lying outside of the approximately 45° angle of the main population. (Figures 6 A-H and 11 A-H). This gate, in addition to the viability, cytokeratin, and MC5 gates, was then applied to the remaining histograms (i.e., 1-parameter DAPI and 2-parameter DAPI/PCNA histograms), and acquisition was restarted. Figures 2 A-H and 7 A-H illustrate ungated histograms. A summary of gating may be found in Bauer et al., Clinical Flow Cytometry, Principles and Applications, Chapter 5, pgs. 87-88 (Williams and Wilkins, 1993).

Histogram Analysis

The Elite software permits the calculation of statistical data on populations defined by both gating and analysis regions (like gating regions, analysis regions are rectilinear regions or bitmaps in a 2-parameter histogram or rectilinear regions in a 1-parameter histogram, encompassing selected populations). The statistics calculated include the number of events within the regions, and the mean channel, peak position and height, standard deviation, and coefficient of variation of the selected populations (on both histogram axes, where applicable), and the percent of total histogram events that are defined by the region. Statistics used in this application for immunofluorescence and viability analysis included the percent "positive" (i.e., the percent of total histogram events defined by analysis regions set above the background fluorescence established by isotype controls, and including approximately 2% of the total events in each of the isotype control histograms) and the ratio of mean channels of positive to negative populations for each parameter. Statistics used in this application for DNA analysis included the mean channels and percent of total events within analysis regions encompassing either the G_0/G_1 peak, the G_2/M peak, or the events in between the two (S-phase). More sophisticated DNA analysis was performed with an off-line DNA analysis program, MULTICYCLE®, purchased from Phoenix Flow Systems, Inc. (San Diego, CA).

All publications cited in this specification are indicative of the level of skill of those in the art to which this application pertains. Each publication is individually incorporated herein by reference in the location where it is cited.

One skilled in the art will appreciate that although specific reagents and conditions are outlined in the following preparations and methods, modifications can be made which are meant to be encompassed by the spirit and

scope of the invention. The preparations and methods, therefore, are provided to illustrate the invention. Such alternate means are to be construed as included within the intent and spirit of the present invention as defined by the following claims.

Claims

1. A method for the identification and analysis of a selected population of cells within a mixed cell population, said method characterized by:

(a) staining a cell sample with a panel of biological probes;

(b) running the sample through a fluorescence measuring instrument;

(c) obtaining a histogram from the results of a fluorescence measuring instrument; and

(d) electronic progressive gating to eliminate non-target and dead cells.

2. The staining of cells according to claim 1, further characterized by:

(a) isolating cells;

(b) staining with viability probe;

(c) staining with tumor associated antigen marker;

(d) incubating in a permeabilization/fixation agent ;

(e) incubating in a dehydration agent;

(f) incubating in a detergent;

(g) staining with proliferation associated antigen marker; and

(i) resuspending sample in DNA dye.

3. The staining of cells according to claim 1, further characterized by:

(a) isolating cells;

(b) staining with viability probe;

(c) staining with secondary antibody, then staining with unconjugated mouse antibody;

(d) staining with tumor associated antigen marker;

(e) incubating in permeabilization/fixation agent;

(f) incubating in a dehydrating agent;

(g) incubating in a detergent;

(h) staining with proliferation associated antigen marker;

(j) resuspending sample in DNA dye.

4. The method according to claim 1, further characterized by said population which are neoplastic cells.

5. The method according to claim 1, further characterized by said population which are solid tumor cells.

6. The method according to claim 1, further characterized by said population which are non-adherent tumor cells.

7. The method according to claim 4, further characterized by said cells which are breast tumor cells.

8. The panel according to claim 1, further characterized by a marker specific to tissue type, a marker associated with the disease state, a proliferation marker, a DNA-specific dye, and a viability marker.

9. The method according to claim 8, further characterized by the viability marker which is a nucleic acid specific dye.

10. The method according to claim 8, further characterized by the viability marker which is an intracellular antigen.

11. The method according to claim 10, further characterized by the intracellular antigen which is tubulin.

12. The method according to claim 8, further characterized by the viability marker which is a cytoskeletal antigen.

13. The method according to claim 12, further characterized by the cytoskeletal antigen which is cytokeratin.

14. The panel according to claim 8, further characterized by MC5-red dye 1, cytokeratin-energy coupled dye, proliferating cell nuclear antigen-allophycocyanin, ethidium monoazide, and 4',6-Diamidino-2-phenylindole.

15. The panel according to claim 8, further characterized by MC5-red dye 1, cytokeratin-energy coupled dye, proliferating cell nuclear antigen-fluorescein isothiocyanate, 7-aminoactinomycin D, and 4',6-Diamidino-2-phenylindole.

16. The panel according to claim 8, further characterized by MC5-allophycocyanin, cytokeratin-red dye 1, proliferating cell nuclear antigen-cyanine 5, Tubulin-SAM-fluorescein isothiocyanate, and 4',6-Diamidino-2-phenylindole.

17. The panel according to claim 8, further characterized by MC5-red dye 1, cytokeratin-energy coupled dye, proliferating cell nuclear antigen-cyanine 5, Tubulin-SAM-fluorescein isothiocyanate, and 4',6-Diamidino-2-phenylindole.

18. The method according to claim 1, further characterized by the fluorescence measuring instrument which is a flow cytometer.

19. The progressive gating of claim 1, further characterized by the sequential application of gates to permit analysis of desired parameters of target cells.

20. The method according to Claim 19, further characterized by the target cells which are neoplastic cells.

21. The method according to Claim 19, further characterized by the target cells which are solid tumor cells.

22. The method according to Claim 19, further characterized by the target cells which are non-adherent tumor cells.

23. The method according to Claim 19, further characterized by the target cells which are breast tumor cells.

24. A method for monitoring drug treatment in cancer therapy which method is characterized by measuring relevant disease associated markers and DNA content by a fluorescence measuring instrument.

25. The method according to claim 1, further characterized by measuring the DNA content and proliferation in cells of a heterogeneous sample.

26. The method according to claims 1, 24 and 25, further characterized by the fluorescence measuring device which is a flow cytometer.

27. The method according to claims 2 and 3, further characterized by the permeabilization/fixation agent which is lysolecithin/paraformaldehyde.

28. The method according to claims 2 and 3, further characterized by the dehydration agent which is methanol.

29. The method according to claims 2 and 3, further characterized by the detergent which is NP40.

30. A kit for the isolation or enrichment of cells for analysis of complex biological tissues, the kit consisting of a panel of markers and including containers of each marker, said panel characterized by:

(a) a viability marker;
(b) a tumor-associated antigen;
(c) a tissue-specific antigen;
(d) a proliferation-associated antigen; and
(e) a DNA-content dye.

31. The panel according to claim 30, further characterized by MC5-red dye 1, cytokeratin-energy coupled dye, proliferating cell nuclear antigen-allophycocyanin, ethidium monoazide, and 4',6-Diamidino-2-phenylindole.

32. The panel according to claim 30, further characterized by MC5-red dye 1, cytokeratin-energy coupled dye, proliferating cell nuclear antigen-fluorescein isothiocyanate, 7-aminoactinomycin D, and 4',6-Diamidino-2-phenylindole.

33. The panel according to claim 30, further characterized by MC5-allophycocyanin, cytokeratin-red dye 1, proliferating cell nuclear antigen-cyanine 5, Tubulin-SAM-fluorescein isothiocyanate, and 4',6-Diamidino-2-phenylindole.

34. The panel according to claim 30, further characterized by MC5-red dye 1, cytokeratin-energy coupled dye, proliferating cell nuclear antigen-cyanine 5, Tubulin-SAM-fluorescein isothiocyanate, and 4',6-Diamidino-2-phenylindole.

35. The kit according to claim 30, further characterized by the viability marker which is a nucleic acid specific dye.

36. The kit according to claim 30, further characterized by the viability marker which is an intracellular antigen.

37. The kit according to claim 36, further characterized by the intracellular antigen which is tubulin.

38. The kit according to claim 30, further characterized by the viability marker which is a cytoskeletal antigen.

39. The method according to claim 38, further characterized by the cytoskeletal antigen which is cytokeratin.

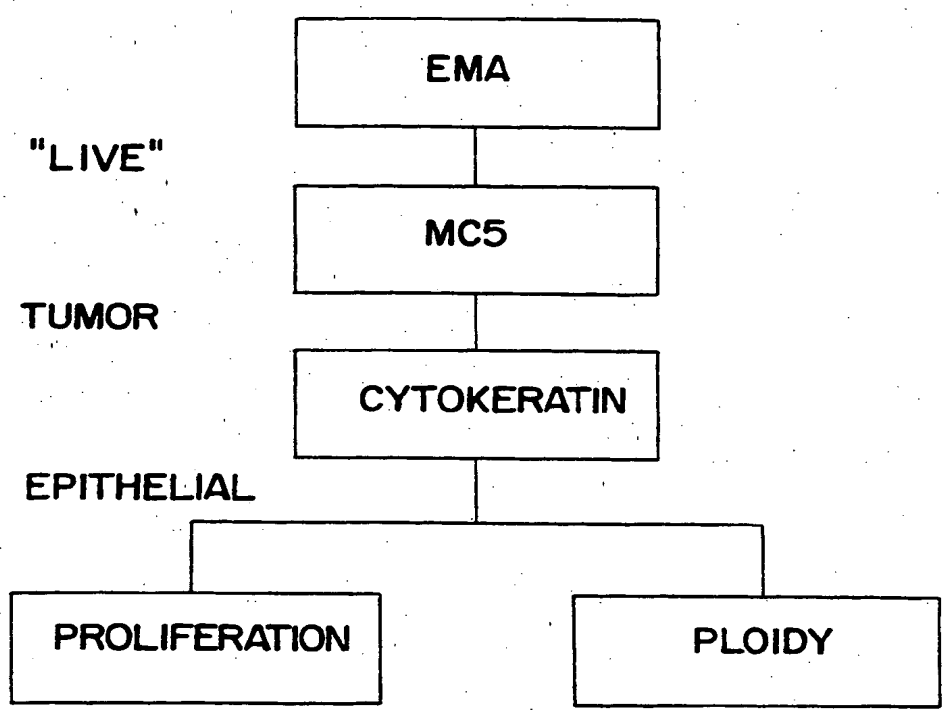


FIG. 1

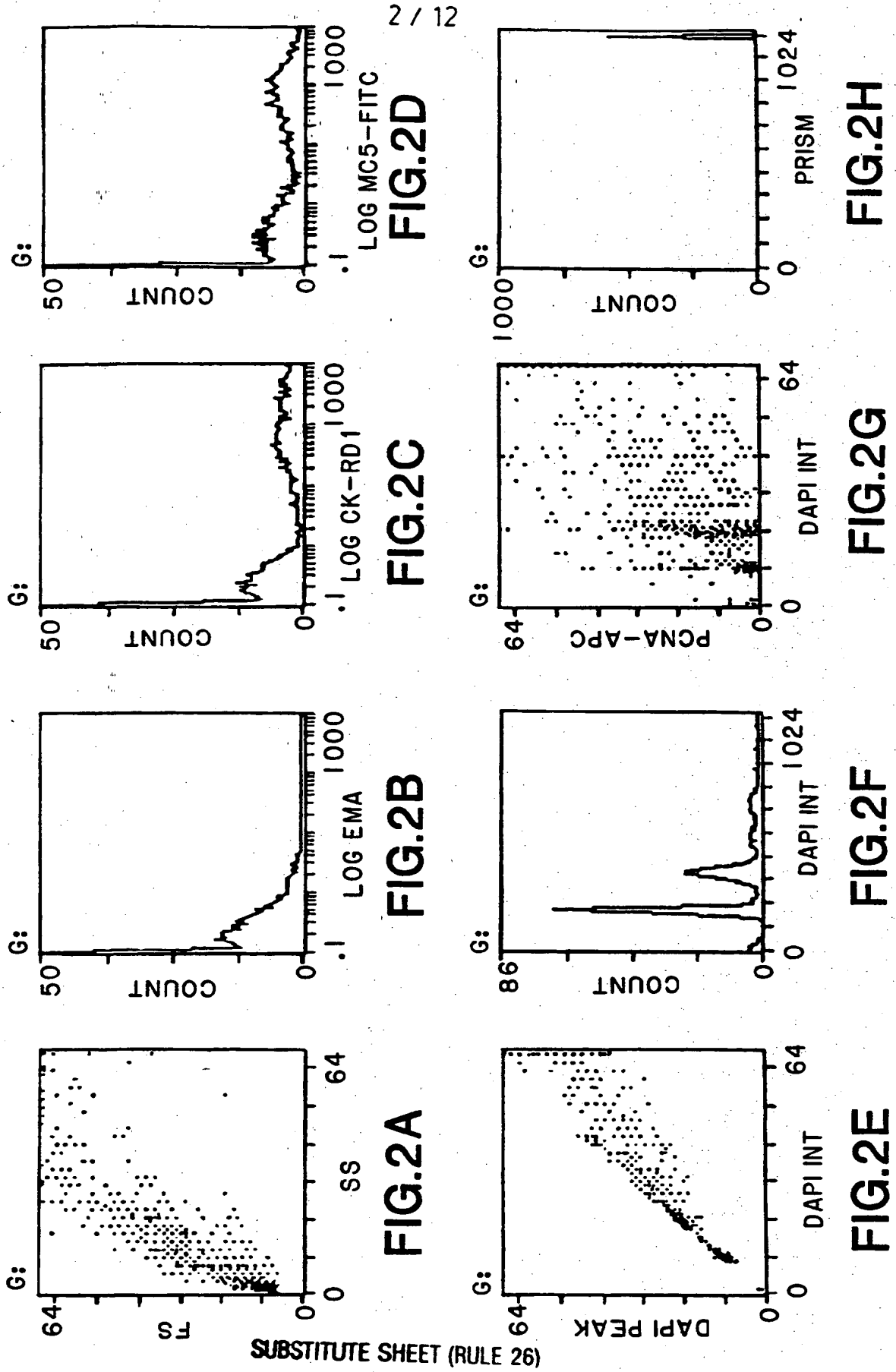


FIG.2A FIG.2E FIG.2B FIG.2F FIG.2C FIG.2G FIG.2D FIG.2H

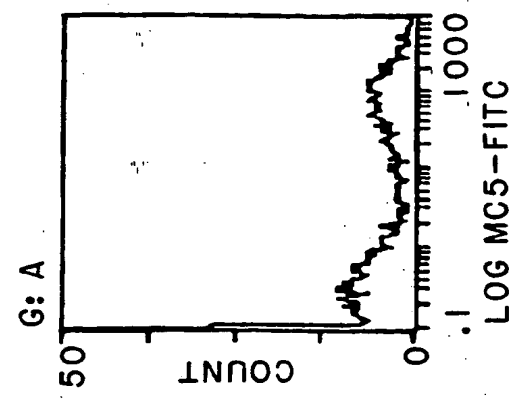


FIG.3D

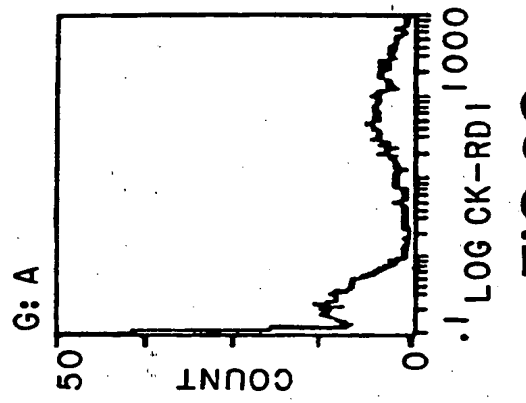


FIG.3C

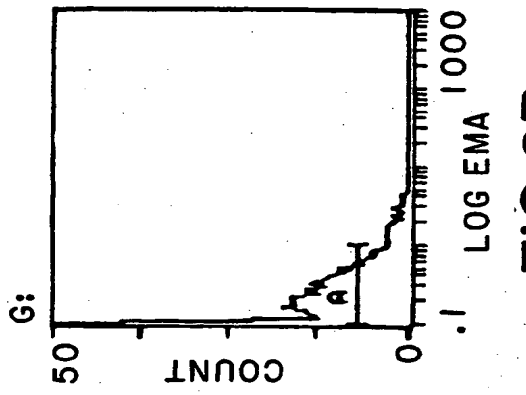


FIG.3B

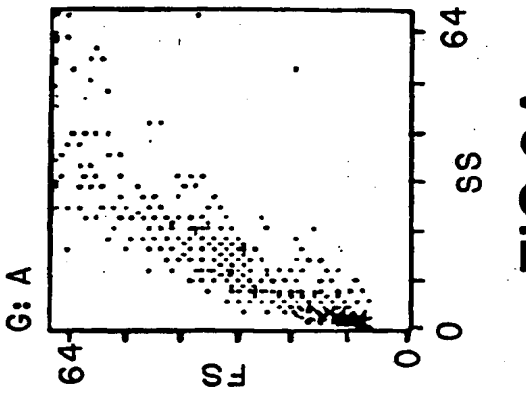


FIG.3A

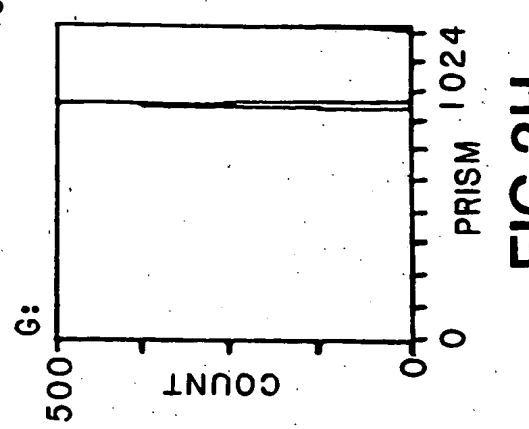


FIG.3H

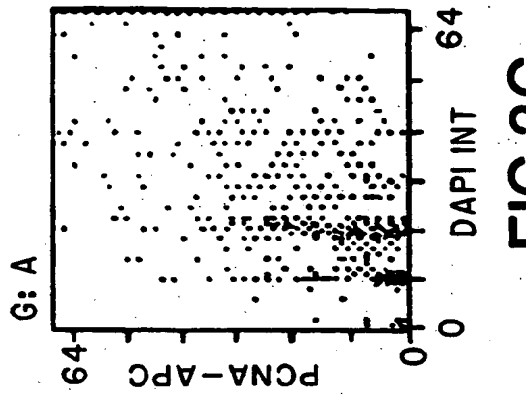


FIG.3G

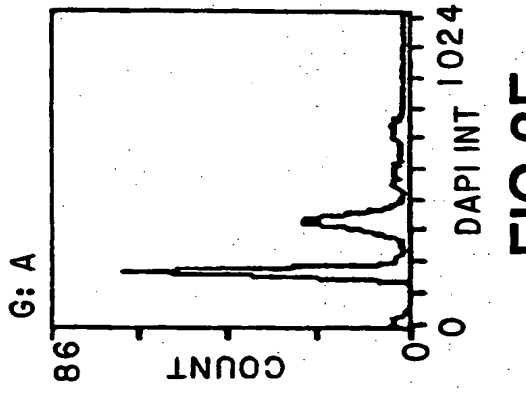


FIG.3F

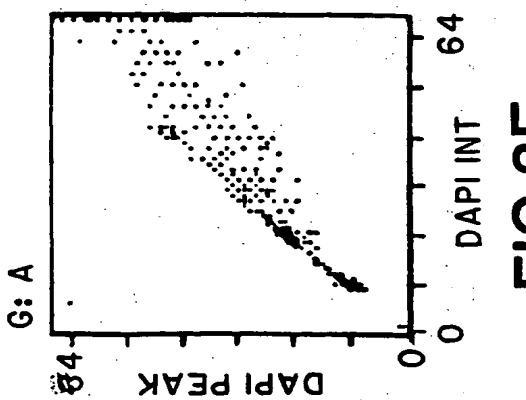


FIG.3E

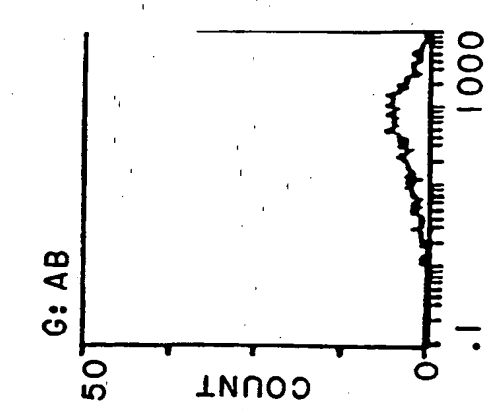


FIG.4D

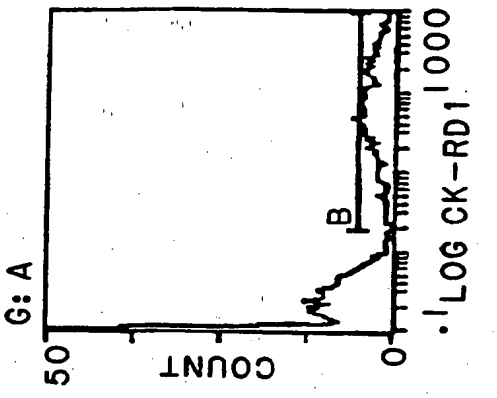


FIG.4C

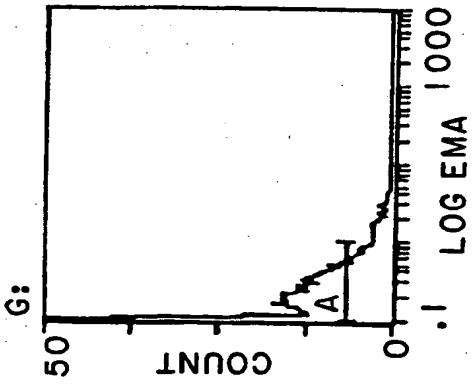


FIG.4B

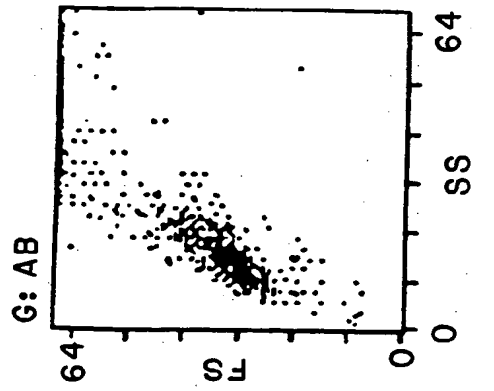


FIG.4A

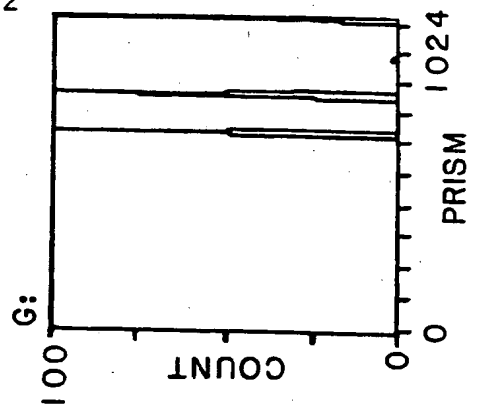


FIG.4H

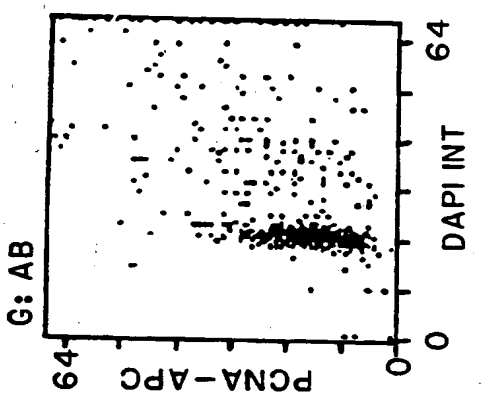


FIG.4G

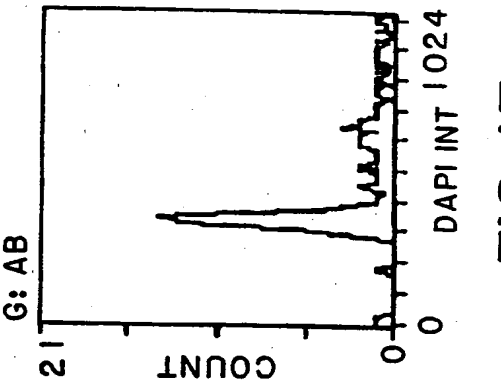


FIG.4F

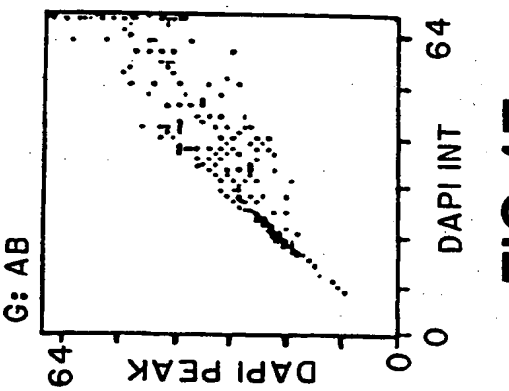


FIG.4E

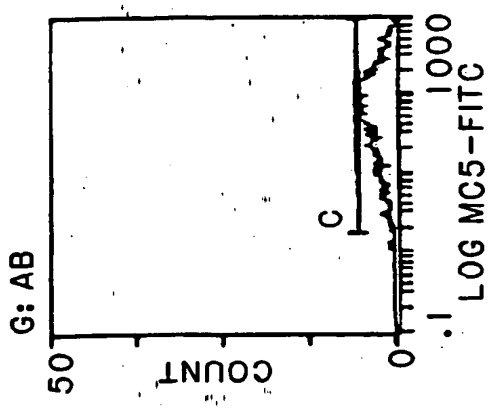


FIG.5D

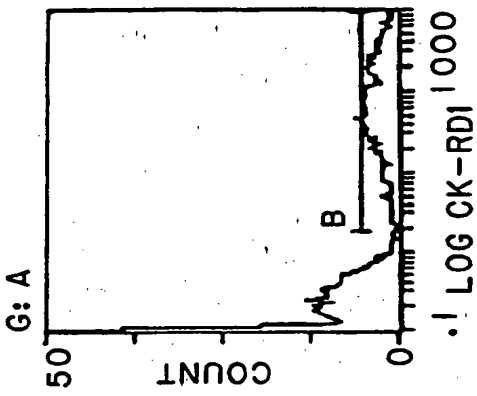


FIG.5C

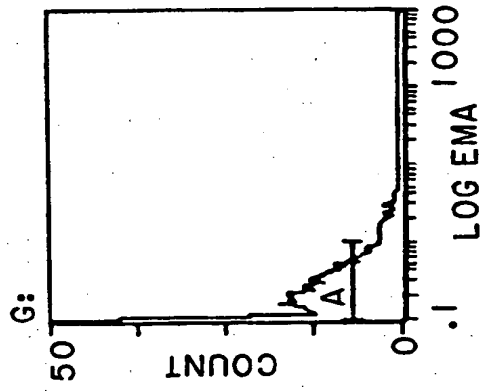


FIG.5B

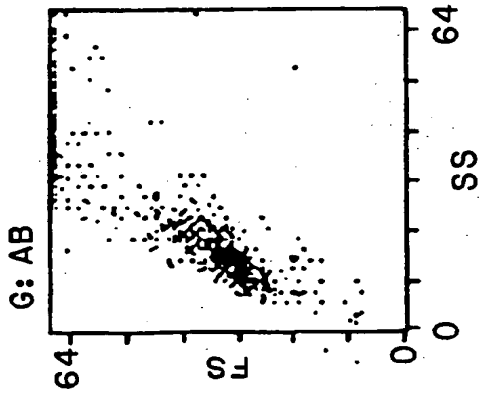


FIG.5A

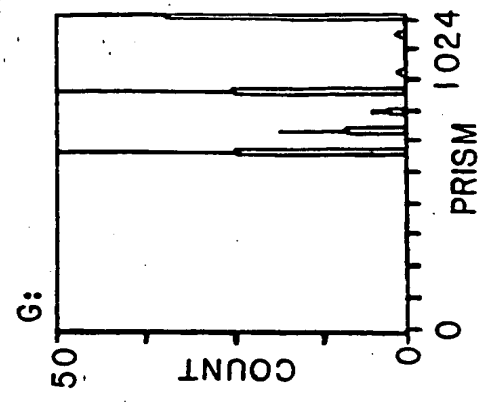


FIG.5H

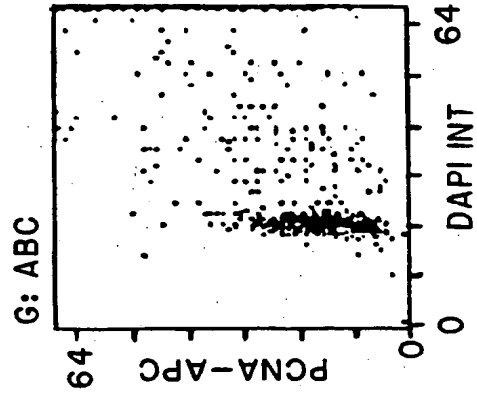


FIG.5G

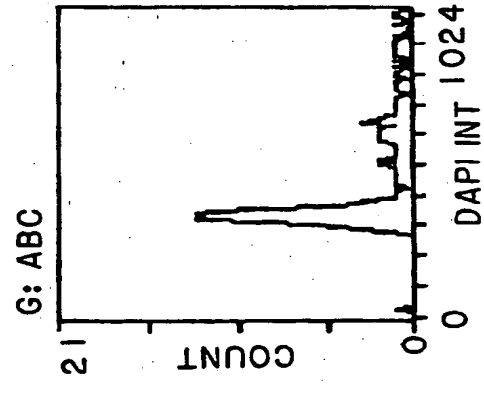


FIG.5F

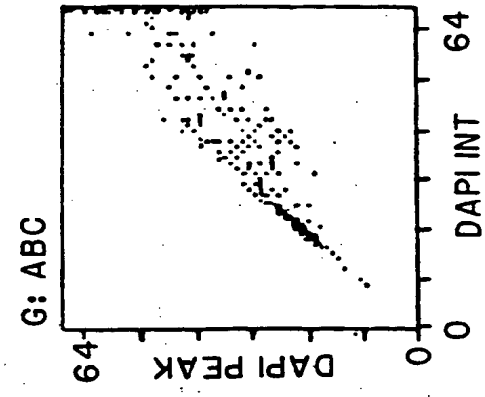
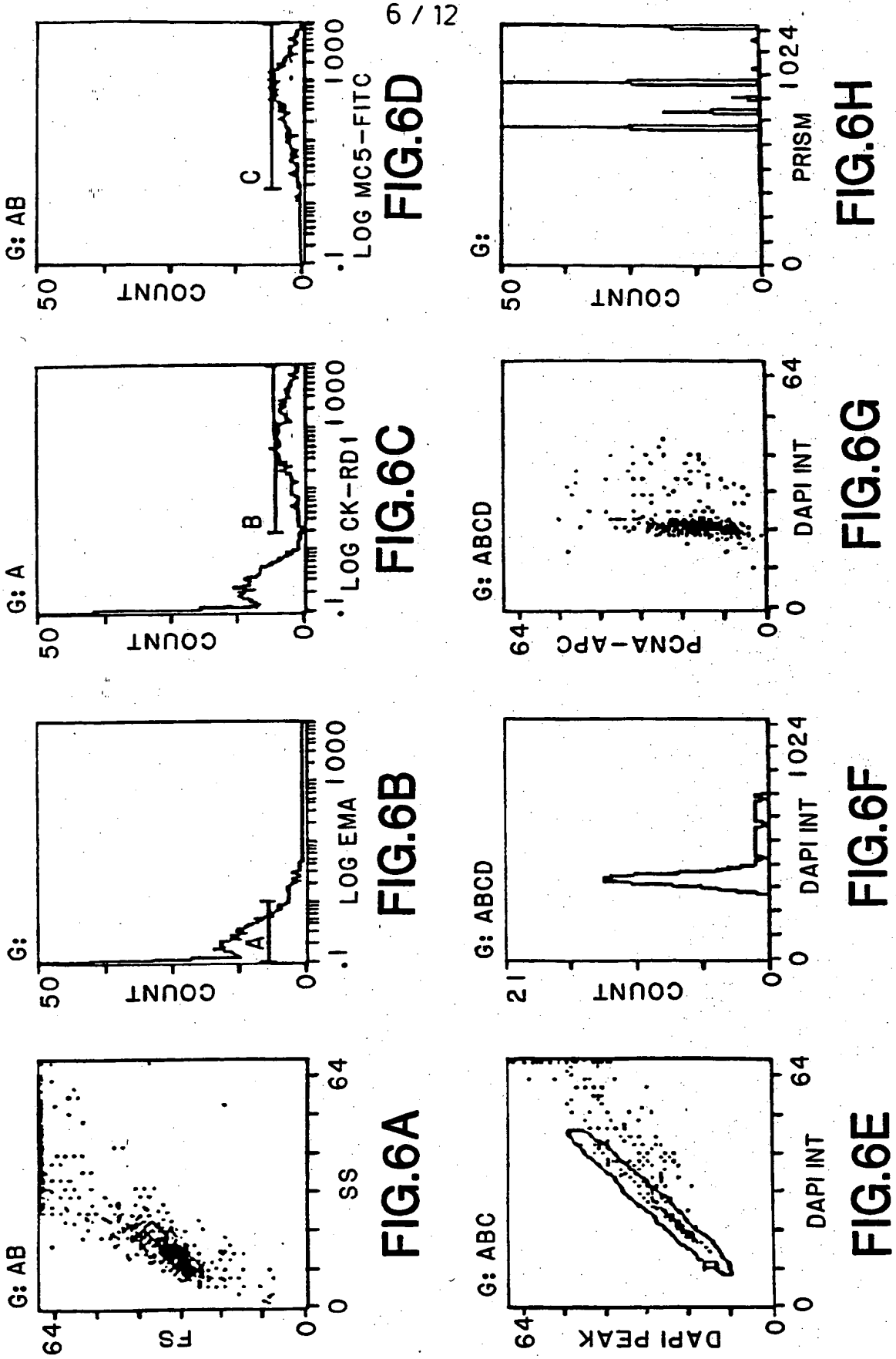
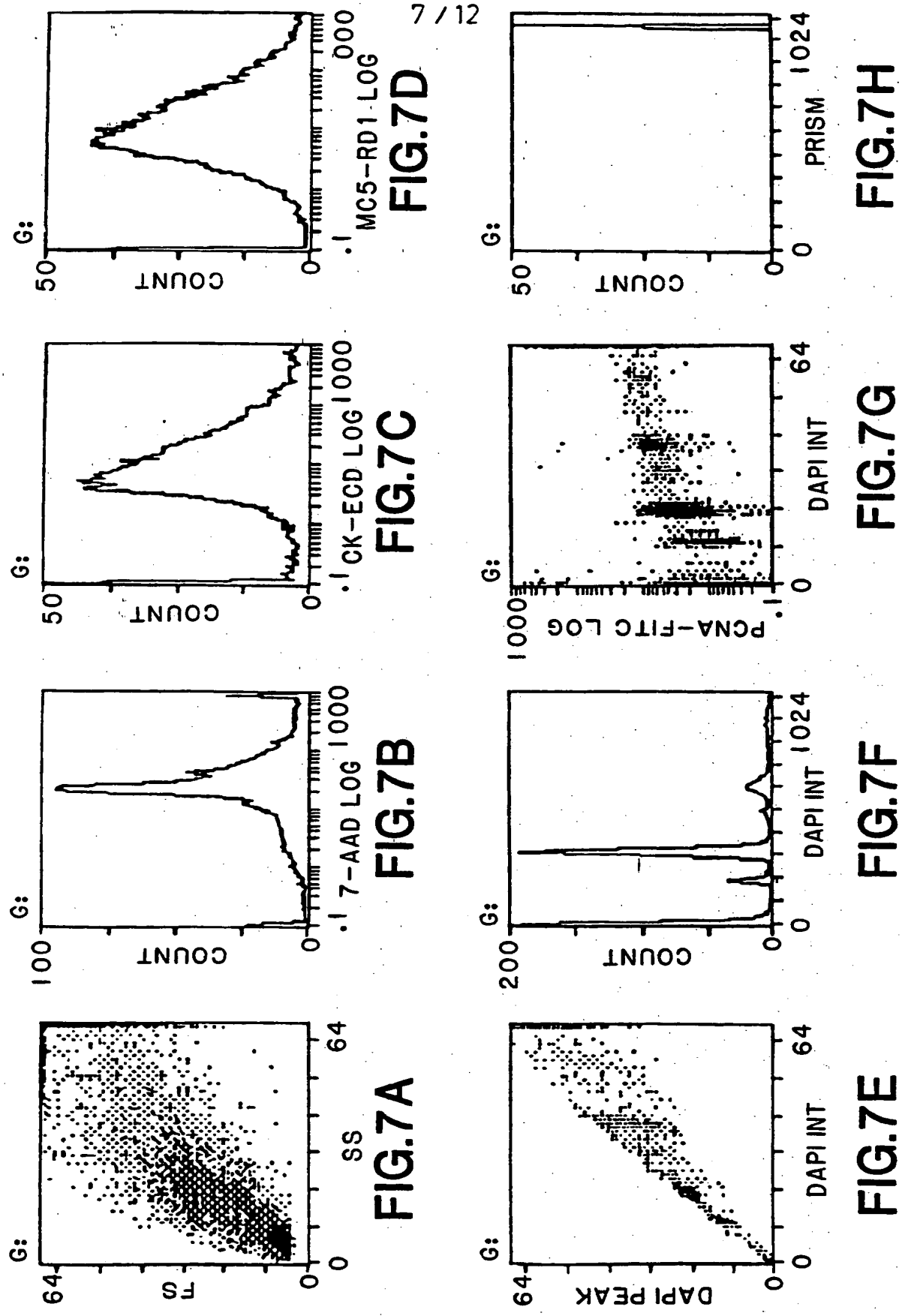


FIG.5E





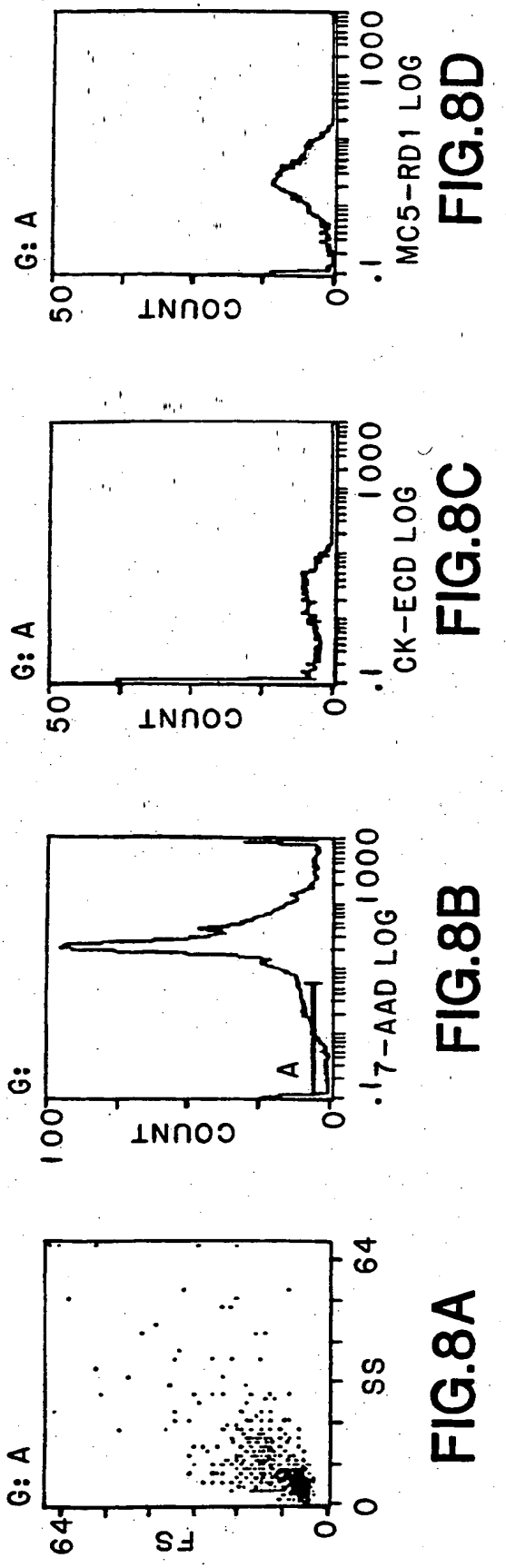


FIG.8D FIG.8H
FIG.8C FIG.8G
FIG.8B FIG.8F
FIG.8A FIG.8E

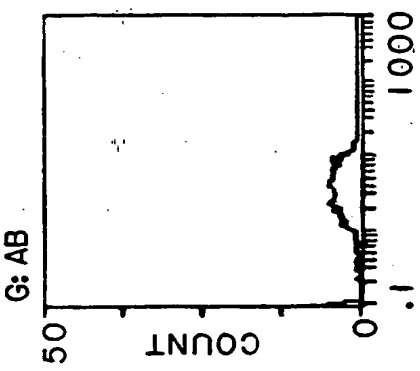


FIG.9D

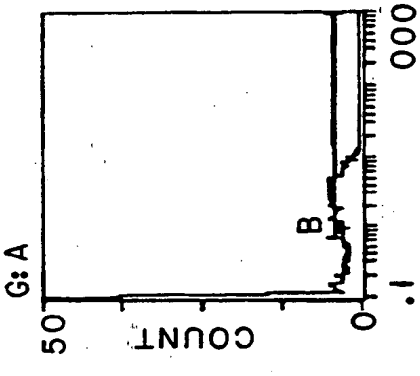


FIG.9C

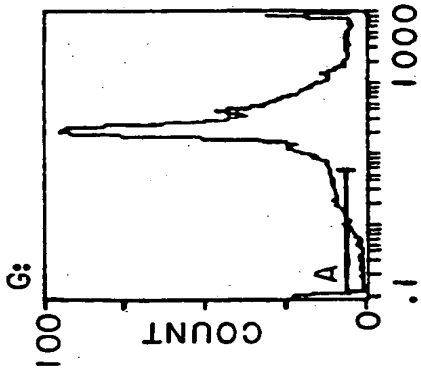


FIG.9B

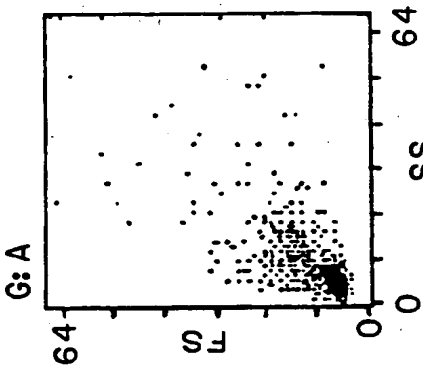


FIG.9A

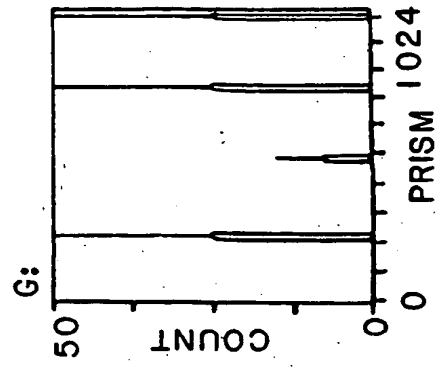


FIG.9H

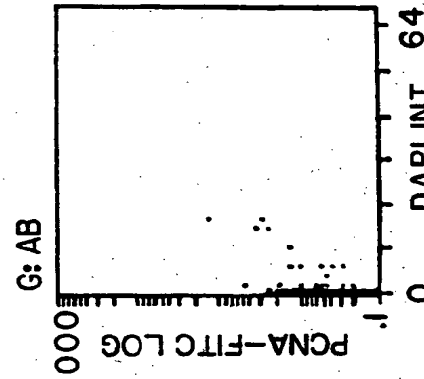


FIG.9G

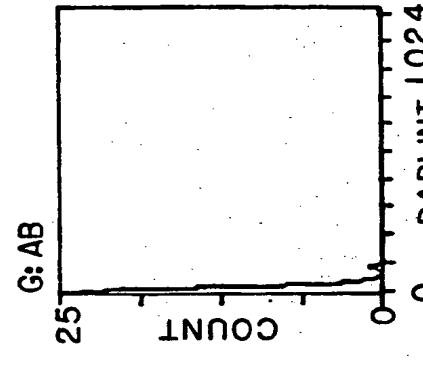


FIG.9F

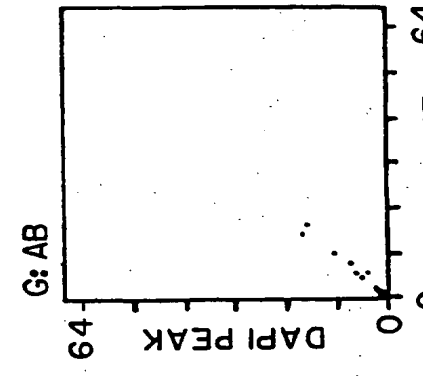


FIG.9E

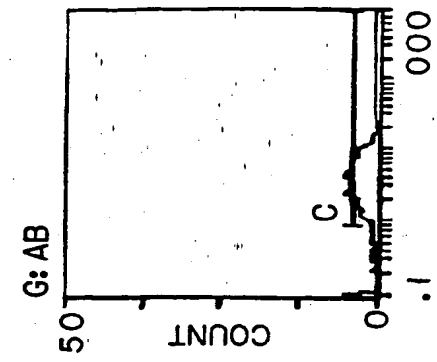


FIG.10D

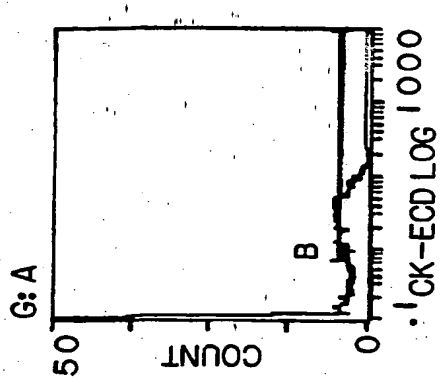


FIG.10C

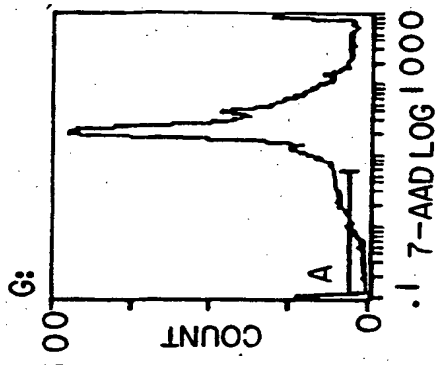


FIG.10B

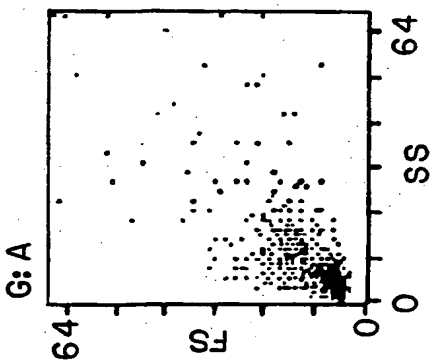


FIG.10A

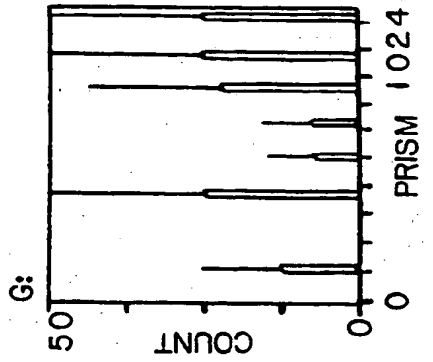


FIG.10H

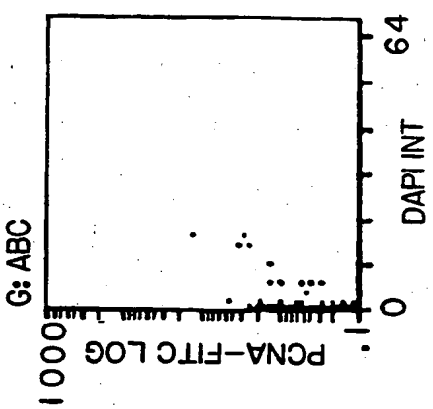


FIG.10G

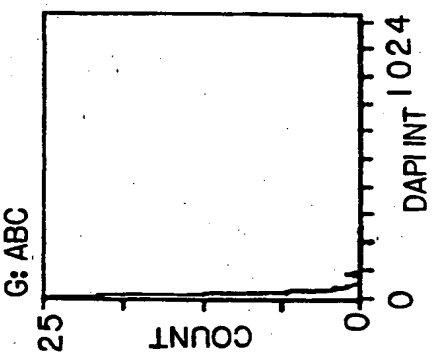


FIG.10F

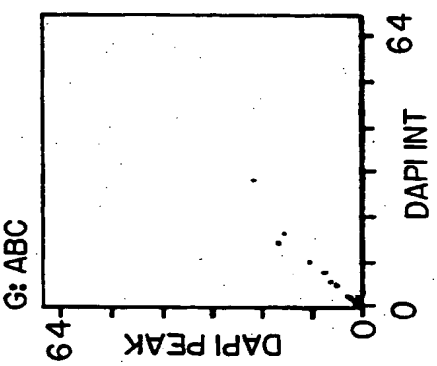


FIG.10E

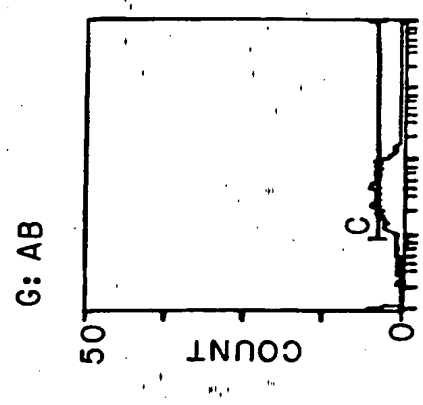


FIG.11D

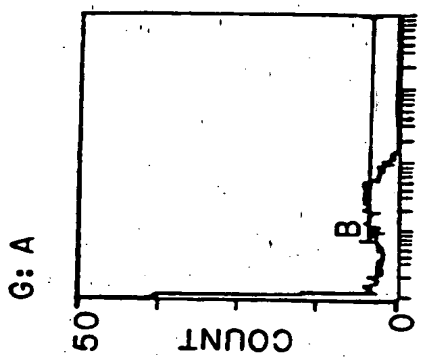


FIG.11C

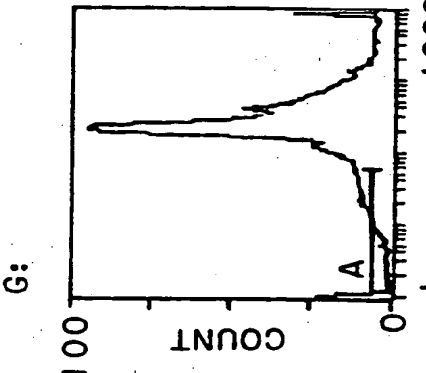


FIG.11B

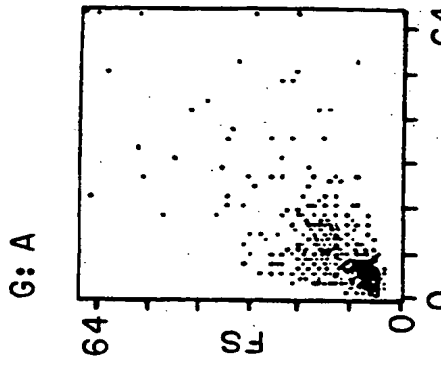


FIG.11A

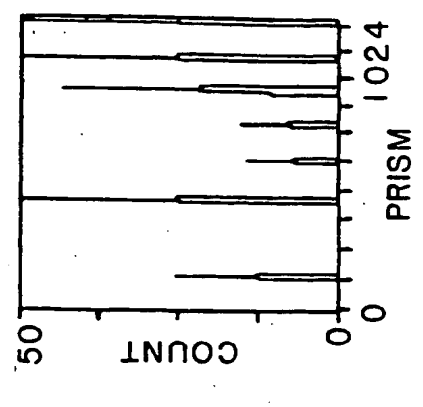


FIG.11H

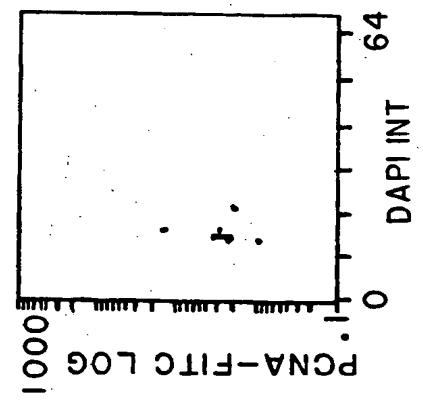


FIG.11G

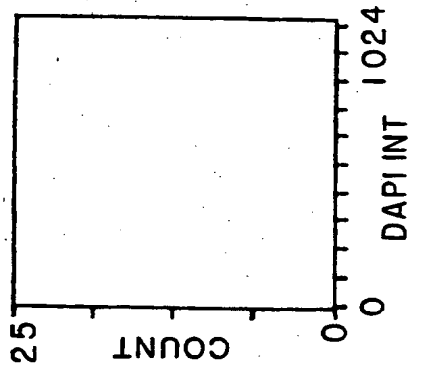


FIG.11F

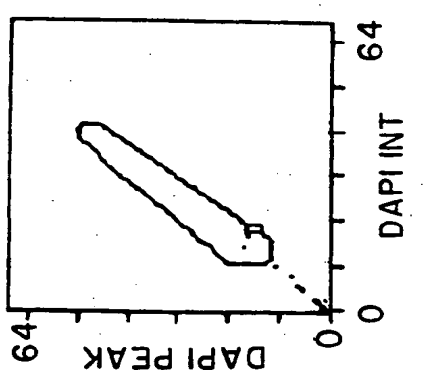


FIG.11E

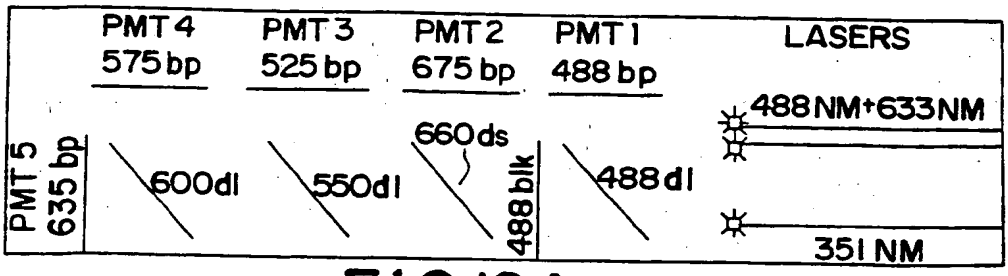


FIG. 12A

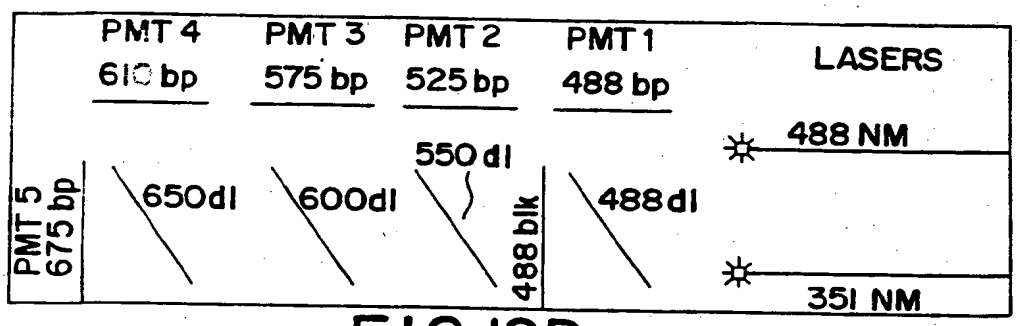


FIG. 12B

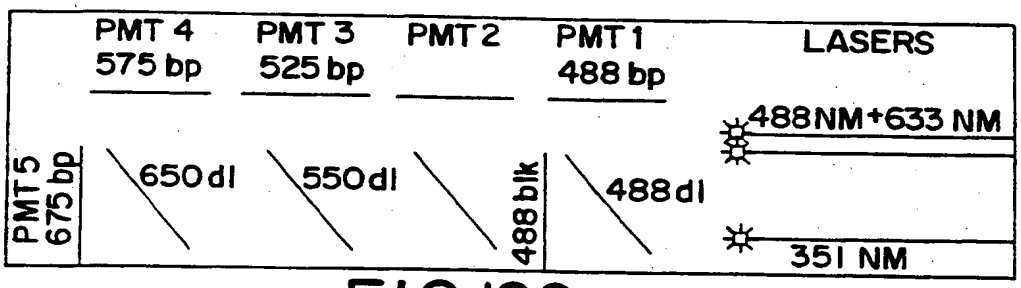


FIG. 12C

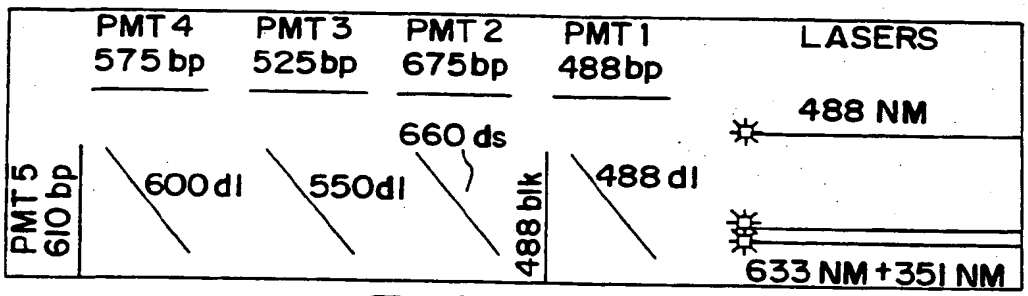


FIG. 12D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/00524

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68; G01N 33/49, 33/533, 33/536, 33/574
US CL :435/2, 6, 7.23, 7.24; 436/64, 172, 536, 546
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/2, 6, 7.23, 7.24; 436/64, 172, 536, 546

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, EMBASE
search terms: viability, PCNA, cytokeratin, tubulin, DNA, tumor, breast cancer

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Breast Cancer Research and Treatment, Volume 22, issued 1992, D.W. Visscher et al, "Comparison of PCNA/cyclin immunohistochemistry with flow cytometric S-phase fraction in breast cancer", pages 111-118, see entire document.	1, 4-7, 18-26 --- 2, 3, 8-17, 27-39
X --- Y	Cytometry, Volume 9, issued 1988, L.W.M.M. Terstappen et al, "Discriminating Between Damaged and Intact Cells in Fixed Flow Cytometric Samples", pages 477-484, see entire document.	1, 18, 19, 25 --- 2-17, 20-24, 26-39

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
10 MAY 1995

Date of mailing of the international search report
18MAY1995

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/00524

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the American Association for Cancer Research, Volume 33, issued March 1992, W. Bolton et al, "Simultaneous multiparametric analysis of tissue directed (Cytokeratin), tumor associated (EMA, CEA), and proliferation associated (PCNA, P120) antigens in breast tumor cells", page 39, abstract number 230, see entire abstract.	1-39
Y	Laboratory Investigation, Volume 62, Number 3, issued 1990, D.W. Visscher et al, "Multiparametric Deoxyribonucleic Acid and Cell Cycle Analysis of Breast Carcinomas by Flow Cytometry", pages 370-378, see entire document.	1-39
Y	Cytometry, Volume 12, issued 1991, H.H. Engelhard III et al, "Simultaneous Quantification of c-*myc* Oncoprotein, Total Cellular Protein, and DNA Content Using Multiparameter Flow Cytometry", pages 68-76, see entire document.	14-17, 31-34